



IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis

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Expression of IL-22 is induced in several human inflammatory conditions, including inflammatory bowel disease (IBD). Expression of the IL-22 receptor is restricted to innate immune cells; however, the role of IL-22 in colitis has not yet been defined. We developed what we believe to be a novel microinjection-based local gene-delivery system that is capable of targeting the inflamed intestine. Using this approach, we demonstrated a therapeutic potency for IL-22-mediated activation of the innate immune pathway in a mouse model of Th2-mediated colitis that induces disease with characteristics similar to that of IBD ulcerative colitis (UC). IL-22 gene delivery enhanced STAT3 activation specifically within colonic epithelial cells and induced both STAT3-dependent expression of mucus-associated molecules and restitution of mucus-producing goblet cells. Importantly, IL-22 gene delivery led to rapid amelioration of local intestinal inflammation. The amelioration of disease by IL-22 was mediated by enhanced mucus production. In addition, local gene delivery was used to inhibit IL-22 activity through overexpression of IL-22-binding protein. Treatment with IL-22-binding protein suppressed goblet cell restitution during the recovery phase of a dextran sulfate sodium-induced model of acute colitis. These data demonstrate what we believe to be a novel function for IL-22 in the intestine and suggest the potency of a local IL-22 gene-delivery system for treating UC.

Introduction

IL-22 belongs to the IL-10 family of cytokines (1–3) and has recently been shown to be preferentially expressed by the Th17 subset (4, 5). IL-22 targets innate immune pathways due to the restricted expression of IL-22 receptors on innate cells, such as epithelial cells, keratinocytes, and hepatocytes but not acquired immune cells, including T or B cells (1–3, 6–9). IL-22 serves as a strong activator of STAT3 (6, 8, 9). Interestingly, IL-22 has been demonstrated to possess the dual abilities of enhancing the expression of regulatory (e.g., SOCS3, IL-10, and antibacterial peptides) (7–10) and inflammatory (e.g., IL-8 and CRP) (8, 11) molecules. Indeed, the role of IL-22 in inflammation differs depending on the specific tissue: e.g., IL-22 contributes to the regulation of hepatitis (6), whereas dermal inflammation is mediated by this cytokine (5).

Inflammatory bowel disease (IBD) is a chronic, relapsing intestinal inflammatory condition that is classified into 2 major forms, Crohn disease (CD) and ulcerative colitis (UC). CD and UC are mediated by both common and distinct mechanisms and exhibit distinct clinical features (12–14). Interestingly, recent studies have demonstrated that colonic IL-22 expression is induced in IBD, but this inducible IL-22 expression is significantly higher in CD as compared with UC (8, 11). IL-22 is capable of enhancing the ERK-mediated expression of a proinflammatory cytokine, IL-8, by

colonic epithelial cell (CEC) lines in vitro (8). In addition, IL-22 is preferentially produced by Th17 cells (4, 5), which have recently been shown to play a pathogenic role in CD-like experimental colitis (15, 16). Therefore, a pathogenic role of IL-22 in CD has been proposed (8, 11). In contrast, a regulatory role of IL-22 in IBD has recently been proposed due to the ability of IL-22 to dampen systemic inflammatory response through the induction of lipopolysaccharide-binding protein (17). Thus, the role of IL-22 in IBD is still unclear and remains to be established.

In this report, we provide unexpected insights into the role of IL-22 that contributes to goblet cell mucus restitution and rapid attenuation of local inflammation associated with Th2-mediated colitis.

Results

Insufficient expression of inducible IL-22 expression in Th2-mediated colitis as compared with Th1-mediated colitis. Through a combined screening approach utilizing DNA microarray and quantitative PCR analysis of an additional 1,300 molecules not covered by the DNA microarray chip, we observed specific induction of IL-22 expression within the colon after the onset of both Th2-mediated colitis in TCR α KO (TCR α KO) mice (18, 19) and Th1-mediated colitis in the CD45RB^{hi} transfer model (20) (Figure 1A). However, the expression levels of IL-22 detected were significantly lower in the TCR α KO mice in comparison with the CD45RB^{hi} transfer model (Figure 1A). This expression pattern of IL-22 was consistent with studies in human IBD wherein IL-22 expression was lower in UC in comparison with CD (8, 11) (Figure 1B). In addition, similar to humans (11), a major source of IL-22 in the inflamed colon of mice was CD4⁺ T cells (Figure 1C). The expression of IL-22 by purified colonic CD4⁺ T cells was significantly

Nonstandard abbreviations used: CD, Crohn disease; CEC, colonic epithelial cell; DOTAP, 1,2-dioleoyl-3-trimethylammonium propane; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; IL-22BP, IL-22-binding protein; LP, lamina propria; Muc, mucus-associated protein; UC, ulcerative colitis.

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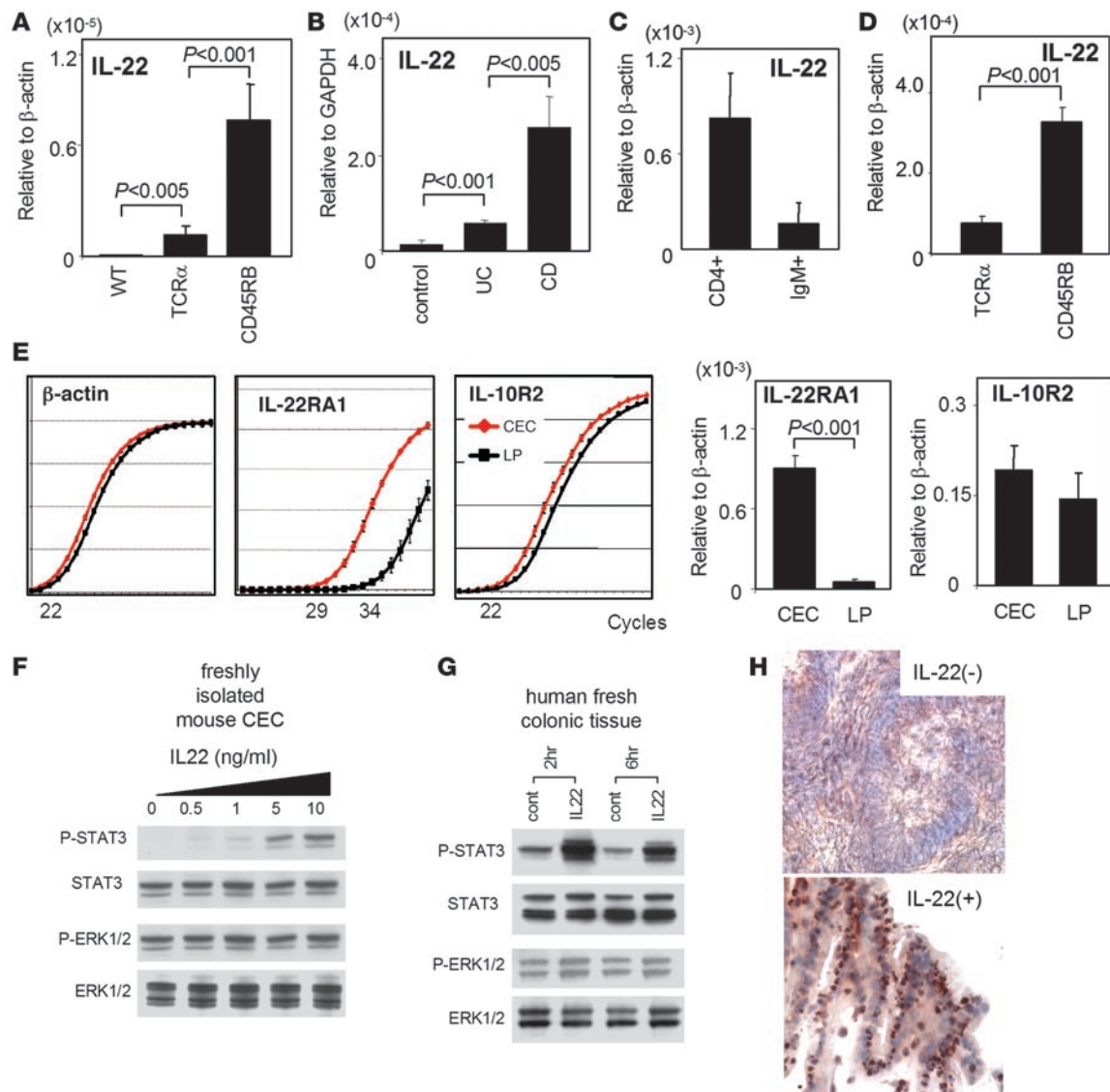


Figure 1

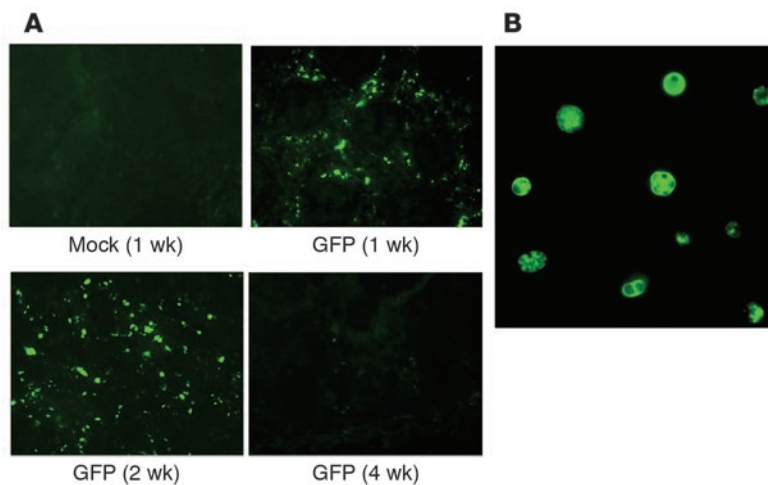
Enhancement of STAT3 activation in CECs by IL-22. **(A)** Expression levels of IL-22 in normal colon of WT ($n = 9$) and inflamed colon of TCR α KO mice ($n = 9$) and the CD45RB colitis model ($n = 4$). **(B)** Expression levels of IL-22 in intestinal biopsy samples from healthy controls ($n = 10$) and from patients with UC ($n = 12$) or CD ($n = 9$) are shown. **(C)** Expression levels of IL-22 in purified CD4 $^+$ T cells and IgM $^+$ cells from inflamed colon of TCR α KO mice are shown. **(D)** Expression levels of IL-22 in purified CD4 $^+$ T cells from inflamed colon of TCR α KO mice and CD45RB model are shown. **(E)** Expressions of β -actin, IL-22RA1, and IL-10R2 in CECs (red lines) and colonic LP (black lines) of TCR α KO mice. Results represent averages of 4 different experiments \pm SEM. Quantitative data relative to β -actin expression are summarized in the 2 right panels. **(F and G)** Freshly isolated CECs from WT mice were stimulated with various doses of IL-22 for 15 minutes **(F)**. Normal human colonic tissues were stimulated without (cont) or with 10 ng/ml of IL-22 **(G)**. Protein lysates (10 μ g) were immunoblotted with anti-phospho-STAT3 (P-STAT3) or anti-phospho-ERK1/2 Abs. After stripping the Abs, the membrane was reprobed with anti-STAT3 or anti-ERK1/2 Abs. The result is representative of 3 individual experiments. **(H)** Human colonic specimen was stimulated without (top panel) or with IL-22 (bottom panel) for 2 hours and subjected to immunohistochemical analysis using anti-phospho-STAT3 Abs. Original magnification, $\times 40$.

lower in TCR α KO mice as compared with the CD45RB hi transfer model (Figure 1D).

IL-22 binds to a heterodimeric receptor that consists of the IL-22RA1 and IL-10R2 chains (2, 3). As previously demonstrated (11), expression of IL-22RA1 was restricted to CECs, including goblet cells of UC and CD patients (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI3194DS1). In addition, IL-22RA1 expression was constitutively detectable on nor-

mal CECs and neoplastic CECs with adenomatous or carcinogenic changes (Supplemental Figure 2). Similar to humans, IL-22RA1 expression was detectable by real-time PCR at high levels in CECs in comparison with the colonic lamina propria (LP) of TCR α KO mice (Figure 1E). In contrast, similar levels of IL-10R2 expression were observed in the CECs and LP of these mice (Figure 1E).

Activation of STAT3, but not ERK1/2, in normal CECs by IL-22. IL-22 has been demonstrated to efficiently activate STAT3 and ERK1/2

**Figure 2**

Development of local gene-delivery system that is capable of targeting colon. (A) A complex of mock or enhanced GFP vector with DOTAP/enhancer reagent was locally microinjected into the proximal part (just below ileocecal junction) of colon of TCR α KO mice through the laparotomy approach. The mice were sacrificed 1, 2, or 4 weeks after the microinjection. Frozen sections of the injection site were subjected to fluorescent microscopic analysis for the detection of GFP signals. The result is representative of 6 individual experiments. (B) Adherent cell populations (such as macrophages and fibroblasts) were isolated from the deepithelialized mucosa with GFP vector delivery using a “walk-out” approach. The obtained cells with an adherent ability were subjected to fluorescent microscopic analysis for the detection of GFP signals. Original magnification, $\times 40$.

in several human epithelial cancer cell lines in vitro (9). This was further confirmed here (Supplemental Figure 3). To determine whether IL-22 activates these signaling cascades in normal CECs, we isolated fresh CECs as crypt units from mice by using the previously validated EDTA perfusion (30 mM) method (21) and stimulated them with IL-22. Similar to human cancer cell lines, IL-22 dose dependently activated STAT3 in the freshly isolated CECs from mice (Figure 1F). In contrast, IL-22 failed to activate the ERK1/2 cascade in the freshly isolated CECs (Figure 1F). To determine whether similar effects were observed in humans, surgically resected human colonic specimens with histologically normal appearance were subjected to organ culture with 5 ng/ml of IL-22. Notably, Western blot analysis showed that STAT3 activation in the colonic specimens was significantly enhanced by stimulation with IL-22 (Figure 1G). In contrast, IL-22 did not enhance the activation of ERK1/2 in the colonic specimens (Figure 1G). Accumulation of phospho-STAT3 in the nucleus of CECs within the IL-22-stimulated human specimens was confirmed by immunohistochemical analysis (Figure 1H). These findings suggest that IL-22 specifically activates STAT3 but not ERK1/2 in normal mouse and human CECs (without neoplastic changes) and are consistent with recent reports showing an inability of IL-22 to activate ERK1/2 in keratinocytes and hepatocytes (10, 17).

Establishment of local gene-delivery approach capable of specifically targeting a selected region within the intestine. Microbial mediated in vivo gene-delivery systems using adenovirus or *Lactococcus lactis* have been used to cause local overexpression of some regulatory molecules in the intestine (22, 23). However, it may be difficult to avoid certain risks in these approaches, e.g., unwanted immune stimulation by virus-like particles or expression of genes of interest throughout the entire intestine by enteric bacteria-mediated gene delivery. To minimize such risks, we tested the efficacy of non-microbial-mediated gene-delivery systems using different agents (e.g., In Vivo GeneSHUTTLE, in vivo MegaFectin, in vivo-jetPEI, TransIT-In Vivo, Chariot, Lipofectamine 2000, and Cellfectin) and different routes for delivery (i.v., intrarectal, and direct microinjection). As a result, we discovered that a pressurized local microinjection of vector/cationic lipid (1,2-dioleoyl-3-trimethylammonium propane-cholesterol [DOTAP-cholesterol])/DNA-condensing agent-2 complexes with 20 mM HEPES (24–26) directly into the colonic mucosa could efficiently deliver the gene of interest

(enhanced GFP) into the injected site. As shown in Figure 2A, large numbers of GFP⁺ cells could be detected in the colonic LP but not CECs within 1 week after gene delivery by this method. GFP signals were detectable in an adherent cell population that was obtained from the deepithelialized mucosa using a “walk-out” approach (27) (Figure 2B). In contrast, GFP signals were not detected in T or B cells (data not shown). Interestingly, few GFP⁺ cells were detectable in the mesenteric lymph nodes and spleen (data not shown). The GFP signal in the colonic LP became undetectable 4 weeks after delivery (Figure 2A). These observations show that inducible gene expression by the method of local gene-delivery system is transient (presumably due to utilization of circularized but not linear DNA) and restricted to the colon. Adherent cell populations such as fibroblasts and macrophages may be the major targets of this in vivo gene-delivery system.

Rapid attenuation of Th2-mediated colitis by local IL-22 gene delivery. IL-22 expression was significantly lower in UC and TCR α KO mice as compared with CD and CD45RB models (8, 11) (Figure 1). We therefore determined whether supplementation of IL-22 expression through our local gene-delivery system contributed to the attenuation or exacerbation of chronic colitis in TCR α KO mice. To do so, lipid complexes with secretion vector carrying full-length mouse IL-22 cDNA or mock vector were injected into the proximal colon (just below the ileocecal junction) of TCR α KO mice selected for the presence of severe colitis as defined by enlarged colonic diameter, as previously described (28) (Figure 3C). These mice were sacrificed 2 weeks after local gene delivery. Local gene delivery of IL-22 but not the mock vector induced a marked increase in IL-22 expression in the injection site within the proximal colon (Figure 3A). In contrast, IL-22 expression in the noninjection site (distal colon) was not affected by local gene delivery (Figure 3A). In addition, local gene delivery of IL-22 vector significantly enhanced STAT3 activation in the CECs of proximal colon (the injected site) but not distal colon (noninjected site) as defined by the levels of phospho-STAT3 (Figure 3B and data not shown). Of note, IL-22 gene delivery led to “prompt” attenuation of colitis at the injection but not the noninjection site, as indicated by a significant reduction in the colonic diameter within 2 weeks after IL-22 gene delivery (Figure 3, C and D). This finding was further confirmed by histological analysis that showed the significant reduction of colonic thicknesses and disease scores in the region where IL-22 gene delivery was performed (Figure 3, E and F).

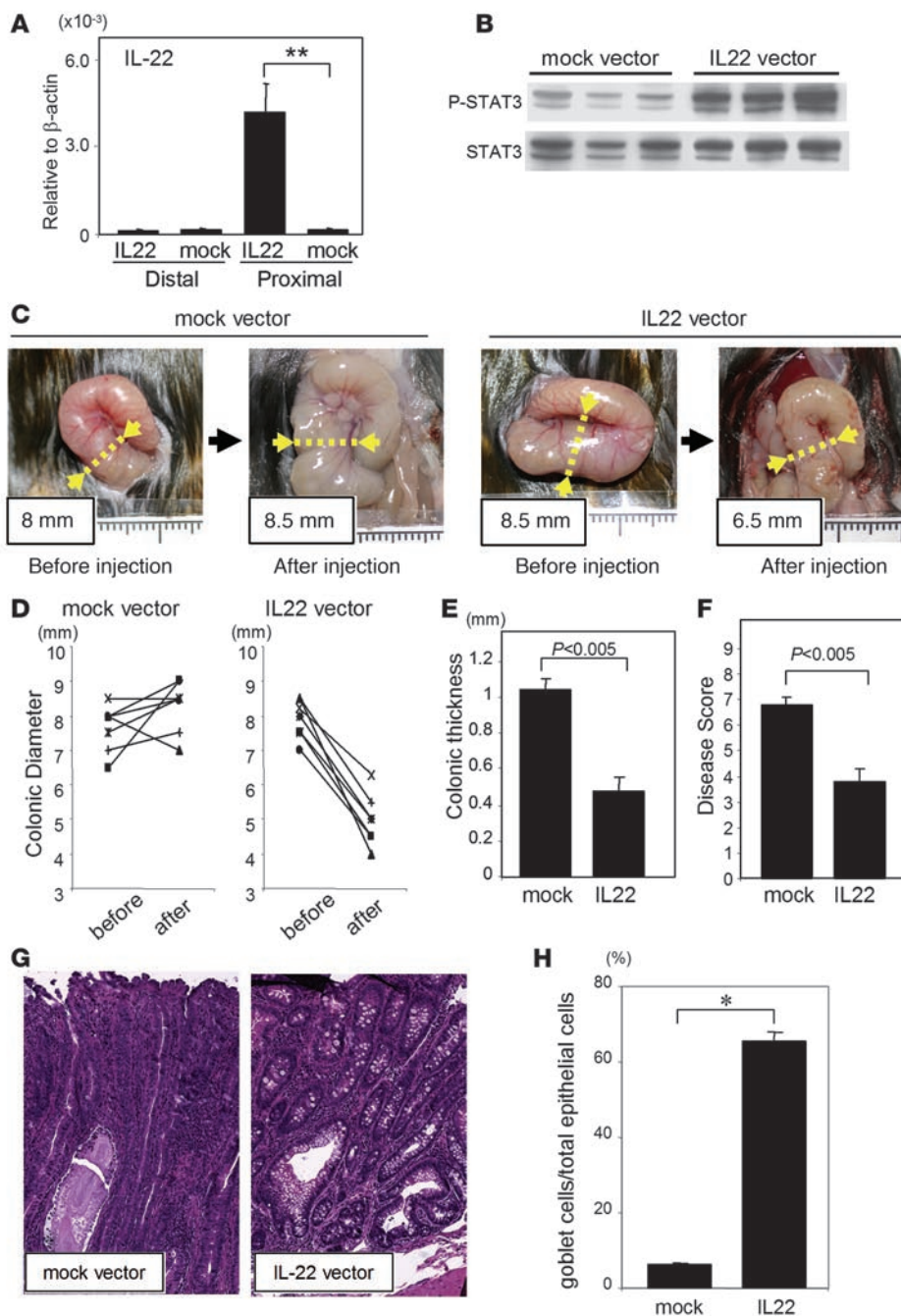


Figure 3

Rapid attenuation of colitis by local IL-22 gene delivery. (A and B) Complexes of IL-22 secretion or mock vector with DOTAP/enhancer reagent were microinjected into the proximal part of colon of TCR α KO mice with colitis. Expressions of IL-22 in the noninjected distal part and injected proximal part are shown in A. Results represent the averages \pm SEM ($n = 6-7$). $**P < 0.005$. Protein lysates from the CECs of the proximal part of colon with mock or IL-22 gene delivery were subjected to immunoblot for evaluation of STAT3 activation (B). (C-H) Laparotomy was carried out on anesthetized TCR α KO mice (24 weeks of age) to confirm the presence of severe colitis as indicated by a marked enlargement of colonic diameter (before injection). Local gene delivery of IL-22 or mock vector into the proximal part (just below the ileocecal junction) was performed in selected TCR α KO mice ($n = 7$ each group) that had severe colitis. Mice were sacrificed 2 weeks after microinjection. IL-22 gene delivery attenuated the inflammation at the injection sites; colonic diameter at the injection sites was markedly reduced in TCR α KO mice that received IL-22 (C, right panels) but not mock (C, left panels) vector delivery. Results are summarized in D. Colonic thickness (E) and disease score (F), which were evaluated by histological examination ($n = 6-7$), are shown. Histology of the proximal colon where gene delivery with mock (left panel) or IL-22 vector (right panel) was received are shown in G. Original magnification, $\times 10$. (H) Percentages of goblet cells among total epithelial cells within the colon where mock ($n = 6$) or IL-22 ($n = 6$) vector delivery were received are shown. $*P < 0.001$.

Goblet cell depletion, which is prominently observed in UC in comparison with CD (12, 29, 30), was observed in the diseased colon of TCR α KO mice treated with mock vector (Figure 3G). Interestingly, IL-22 gene delivery induced a significant restoration of goblet cell expression in TCR α KO mice (Figure 3, G and H). These data suggest that IL-22 contributes to rapid amelioration of chronic colitis in association with goblet cell restitution in TCR α KO mice.

Induction of STAT3-mediated mucus-associated protein expression by IL-22. Supplementation of IL-22 expression in the inflamed colon of TCR α KO mice by local gene delivery significantly reverted goblet cell depletion typically observed in TCR α KO mice (Figure 3, E and F). Therefore, to determine whether IL-22 possesses an ability to

directly stimulate goblet cells, the expression of goblet cell-associated genes in freshly isolated CECs from WT mice without colitis was examined after IL-22 gene delivery. Interestingly, real-time PCR analysis showed that inducible IL-22 overexpression stimulated CECs to express MUC1, -3, -10, and -13, which are involved in mucus layer formation (Figure 4A). Western blot analysis further confirmed that STAT3 activation as well as MUC1 production in WT CECs was induced by local gene delivery of the IL-22-encoding but not mock vector (Figure 4B). In addition, like mouse CECs, IL-22 enhanced the expression of MUC1, -3, and -13 in a human T84 CEC line (Figure 4C). Western blot analysis confirmed that MUC1 production was enhanced after stimulation of T84 cells

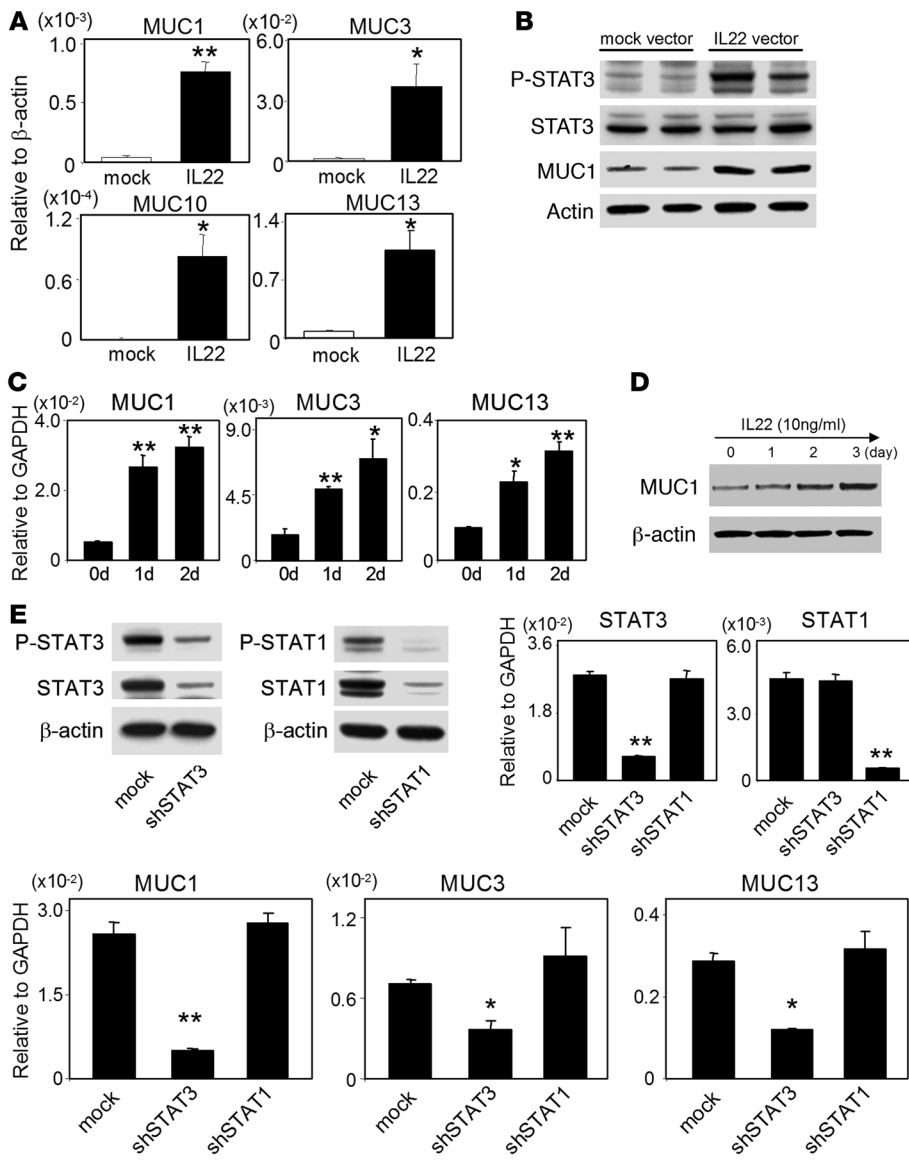


Figure 4
 IL-22-mediated induction of a series of Muc expressions in CECs through STAT3 activation. (A and B) CECs were freshly isolated from WT mice that had received local gene delivery of mock (white bars, n = 5) or IL-22 secretion (black bars, n = 5) vector into the proximal colon. Expressions of MUCs by CECs are shown in A. Protein lysates from freshly isolated CECs were subjected to immunoblot with anti-phospho-STAT3 (P-STAT3) or anti-MUC1 (MUC1) Abs (B). After stripping the Abs, the membrane was reprobed with anti-STAT3 (STAT3) or actin Abs. (C and D) Average expressions of MUCs by T84 cells stimulated with IL-22 (10 ng/ml) in duplicates of 3 individual experiments are shown in C. Protein lysates were immunoblotted with anti-MUC1 Abs (D). After stripping the Abs, membrane was reprobed with β -actin Abs. (E) T84 cells were transfected with a combination of STAT3 shRNA vectors (pKD-STAT3-v2 and -v3) or STAT1 shRNA vectors (pKD-STAT1-v1 and -v2) using Amaxa, which has been shown to efficiently transfect the gene of interest into cells resistant to typical transfection approaches. Cell line with mock vector transfection was used as control. After stimulation of the shRNA- or mock vector-transfected cells with IL-22 (10 ng/ml), the efficiency of the combined shRNA on the silencing of STAT3 or STAT1 expression was confirmed by Western blot (upper left panels) and real-time PCR analyses (upper right panels). Expression of MUC1, -3, and -13 by shRNA (STAT3 or STAT1) or mock vector-transfected cells stimulated with IL-22 was evaluated by real-time PCR analysis. Data represent the average of 3 individual experiments. **P < 0.005; *P < 0.05.

with IL-22 in a time-dependent manner (Figure 4D). To determine whether STAT3 is required for the IL-22-mediated enhancement of mucus-associated protein (Muc) production, we initially optimized the conditions in order to sufficiently inhibit STAT3 expression in the T84 cell line by employing a Nucleofector approach to introduce combined STAT3-shRNA vectors. Inhibition of STAT3 resulted in a significant decrease in IL-22-mediated MUC1, -3, and -13 expression by the T84 cell line (Figure 4E). A capability of IL-22 to stimulate STAT1 activity has previously been demonstrated (17). Therefore, we next tested to determine whether STAT1 is involved in the IL-22-mediated production of MUCs. However, inhibition of STAT1 did not affect MUC production by the T84 cells line (Figure 4E). These findings identify IL-22 as a unique cytokine capable of enhancing mucin production by CECs through the activation of STAT3. Indeed, STAT3 has been demonstrated to interact with a promoter region within the MUC1 gene (31).

The major Th17 cytokine IL-17A has previously been demonstrated to stimulate airway epithelial cells to produce Muc5 (32).

Therefore, to determine whether, similarly to IL-22, IL-17 is also involved in Muc production in the intestine, gene delivery of IL-17A was performed in the colon of WT mice. However, gene delivery did not enhance the activation of STAT3 or the expression of Muc1, -3, -10, or -13 in CECs (Supplemental Figure 4). In addition, Muc5 was undetectable in CECs. These findings suggest the physiologically different functions of IL-22 versus IL-17 in the intestine.

Enhanced mucus production plays a crucial role in IL-22-mediated amelioration of chronic colitis in TCR α KO mice. Since Muc family members form a static external barrier along the epithelial cell surface (33, 34), we determined whether the rapid attenuation of chronic colitis induced by supplementation of IL-22 expression was mediated by the enhanced Muc expression observed. To do so, IL-22 gene delivery was performed in the inflamed colons of TCR α KO mice with or without mucolytic treatment to remove colonic mucus layer. For the mucolytic treatment, a mucolytic agent, N-acetyl-L-cysteine (20 μ g/h), was continuously supplied for 14 days in the colonic lumen through a catheter connected to an ALZET osmotic

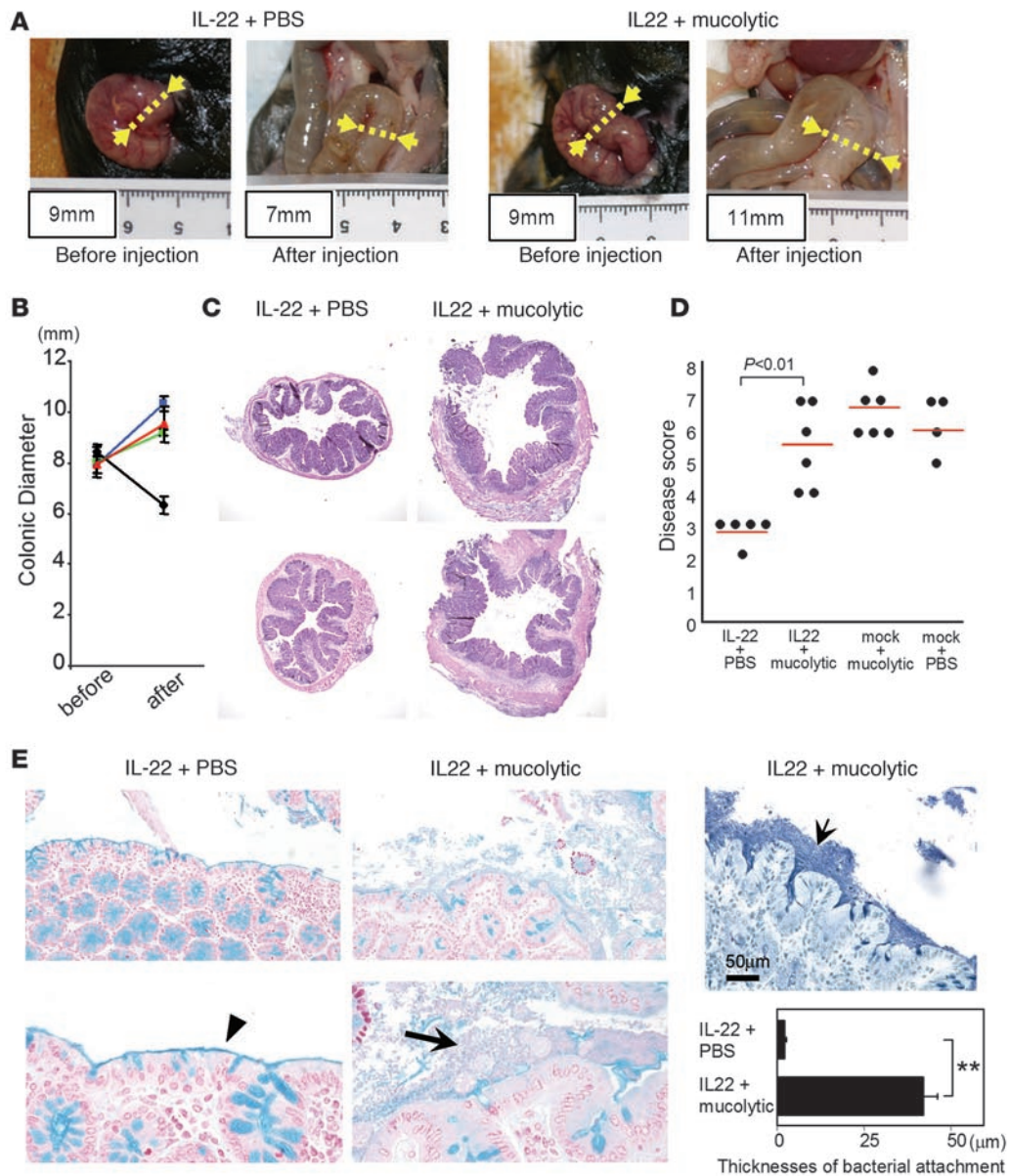


Figure 5

Enhanced mucus production mediates IL-22-induced rapid attenuation of UC-like disease. (A–D) Laparotomy was carried out on anesthetized TCR α KO mice (24 weeks of age) to confirm the presence of colitis as indicated by a marked enlargement of colonic diameter (A, before injection). Local gene delivery of IL-22 vector into the proximal part (just below the ileocecal junction) was performed in the diseased TCR α KO mice. PBS (A, left panels) or mucolytic agent (A, right panels) was continuously administered into the cecal lumen through osmotic pump for 2 weeks. The mice were sacrificed 2 weeks after the microinjection (A, after injection). Numbers in panels indicate the colonic diameter. Summary of change in colonic diameter of 4 mouse groups ($n = 4-6$) (IL-22 gene delivery plus PBS treatment, black; IL-22 gene delivery plus mucolytic treatment, red; mock gene delivery plus mucolytic treatment, blue; and mock gene delivery plus PBS treatment, green) is shown in B. Histology of the colon from IL-22-gene-delivered TCR α KO mice with PBS (C, left panels) or mucolytic agent (C, right panels) and summarized disease score (D) are shown. (E) Alcian blue staining shows preserved mucus layer (blue liner, arrowhead) along epithelial surface of IL-22-gene-delivered TCR α KO mice (left panels). In contrast, mucolytic treatment impaired the mucus layer formation with significant adhesion of enteric bacteria (arrow; middle panels). Adhesion of enteric bacteria was confirmed by toluidine blue staining (top right panel, arrow). Average (randomly selected 20 fields/each mouse of 4 mice) of thicknesses of bacterial layer attached to epithelial surface is summarized in bottom right panel. ** $P < 0.001$. Original magnification, $\times 1$ (C); $\times 10$ (E, top left panels); $\times 40$ (E, bottom left panels).

pump that was implanted within the dorsa of mice. The dose used has been optimized so as not to induce systemic side effects (35). Interestingly, local IL-22 gene delivery failed to attenuate the colitis (as judged by colonic diameter) in TCR α KO mice that were treated

with the mucolytic agent (Figure 5, A and B). Histological examination confirmed that TCR α KO mice that received IL-22 gene delivery plus mucolytic treatment in fact developed more severe colitis compared with mice that received IL-22 gene delivery plus

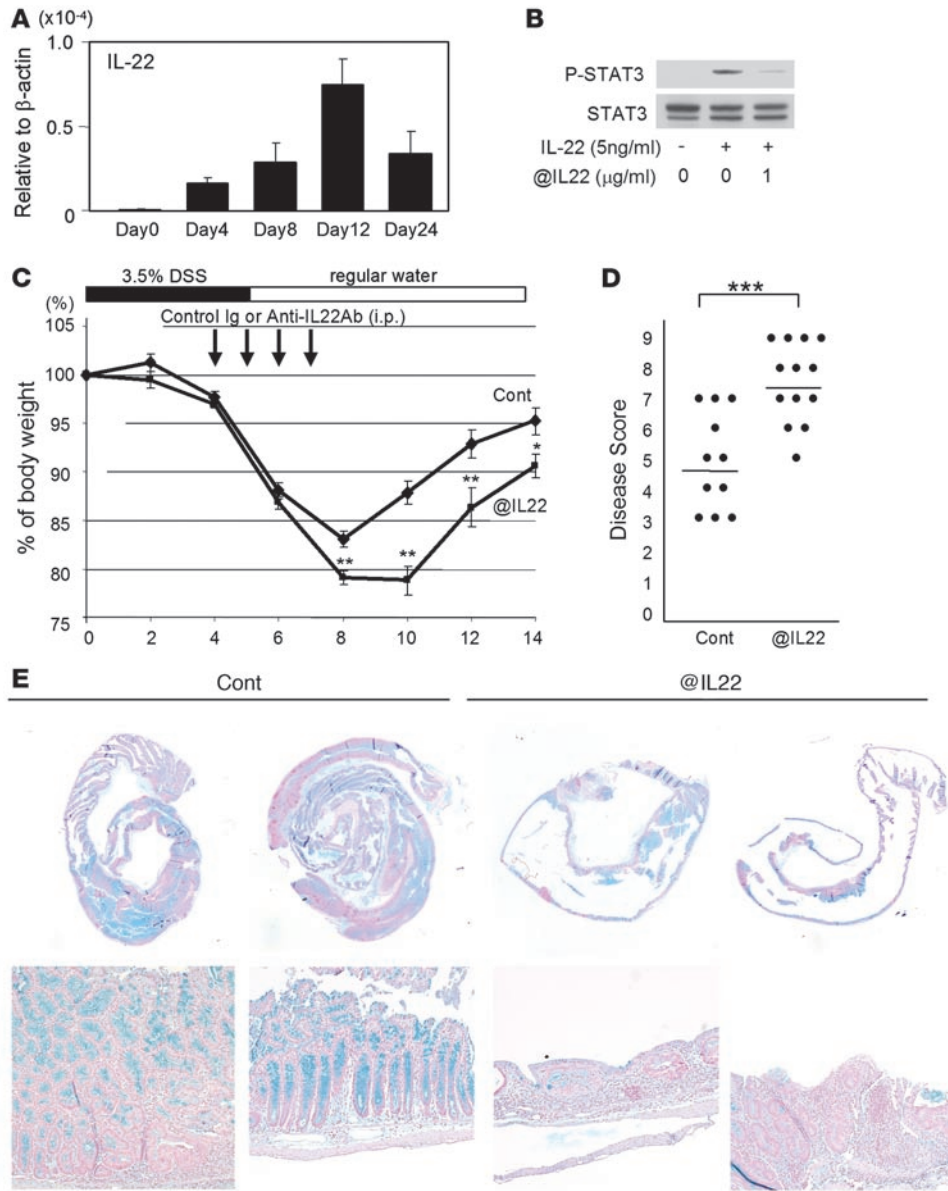


Figure 6

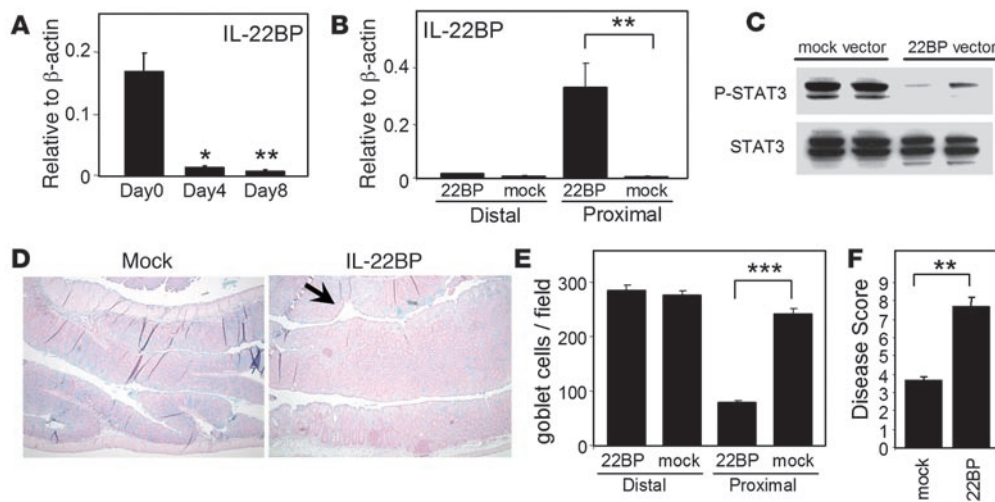
Role of IL-22 in the restitution of goblet cells during recovery from acute intestinal injury. (A) DSS was administered into WT mice for 5 days and the treatment terminated to induce recovery phase. Expression levels of IL-22 (ratio of IL-22/ β -actin) in the colonic LP at days 0 (normal), 4 (acute phase), 8, 12 (recovery phases), and 24 are shown ($n = 4$ each group). (B) CECs were freshly isolated as crypt units from WT mice and stimulated with IL-22 (5 ng/ml) in the presence or absence of anti-IL-22 Abs. Protein lysates (10 μ g) from the stimulated CECs were subjected to immunoblot with anti-phospho-STAT3 (P-STAT3) Abs. After stripping Abs, the membrane was probed with anti-STAT3 Abs (STAT3). (C–E) WT mice were treated with 3.5% DSS in drinking water for 5 days. Repeated i.p. administration of 1.5 mg/injection of anti-IL-22 Abs (@IL22; $n = 12$) or control Ig (Cont; $n = 12$) was performed at days 4, 5, 6, and 7 in selected mice that showed similar body weight loss at day 4 as compared with initial body weight. Body weight change (C), “Swiss rolls” (D, upper panels), and high magnification (D, bottom panels) of colons stained with Alcian blue are shown. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$. Original magnification, $\times 1$ (E, top row); $\times 20$ (E, bottom row). Disease scores are summarized in E.

mock treatment (Figure 5, C and D). Notably, a preserved mucus layer, as evaluated by Alcian blue staining, was detectable along the surface of the colonic epithelium in TCR α KO mice that received IL-22 gene delivery (Figure 5E). In contrast, mucolytic treatment significantly impaired the structure and expression of the mucus layer (Figure 5E). Interestingly, the impaired mucus layer was associated with markedly enhanced adhesion of enteric bacteria to the epithelial cell surface (Figure 5E). These findings suggest that the increased production of Muc, which contributes to preserve mucus layer formation, is involved in the rapid attenuation of established chronic colitis in TCR α KO mice, which is mediated by IL-22.

Contribution of IL-22 to facilitate goblet cell restitution in intestinal inflammation. A useful model to study disease-associated CEC homeostasis is the dextran sulfate sodium-induced (DSS-induced) colitis model, in which acute intestinal inflammation with epithelial loss is induced by treatment with DSS. After termination of the DSS treatment, the acute colitis spontaneously recovers, with

marked CEC regeneration with goblet cell restitution (21). Interestingly, we observed that IL-22 expression in the colon was significantly upregulated during the recovery phase (after termination of DSS treatment) of DSS colitis (Figure 6A). Therefore, to study the role of IL-22 in this colitis model, we neutralized IL-22 activity in vivo during the recovery phase by administering anti-IL-22 Abs that recognize a binding site within IL-22 to IL-10R2 (36) and are indeed capable of inhibiting IL-22-induced activation of STAT3 in CECs (Figure 6B).

Treatment with the anti-IL-22 Abs significantly delayed the recovery from DSS-induced acute colitis as judged by body weight loss (Figure 6C), a widely used measure of injury in DSS colitis (37). In addition, histological analysis using “Swiss rolls” of entire colon showed that the anti-IL-22-treated group exhibited thin colonic wall as compared with the control IgG-treated group (Figure 6D). Interestingly, a strong accumulation of goblet cells, which is generally associated with regeneration of CECs during the recovery phase

**Figure 7**

Effect of IL-22BP on the suppression of IL-22 activity in vivo. (A) Expression levels of IL-22BP (ratio of IL-22BP/ β -actin) in the colonic LP at days 0 (normal), 4 (acute phase), and 8 (recovery phase) of DSS colitis are shown. (B–F) Local IL-22BP gene delivery was performed in the proximal part of the colon, and the mice were subsequently treated with 3% DSS on the third day after the delivery. DSS treatment was terminated at day 5 after initiation, and mice were sacrificed at day 8. Expression of IL-22BP in the distal (noninjected site) and proximal (injected site) colons of mice with mock or IL-22BP gene delivery is shown (B). CECs were freshly isolated from DSS-treated WT mice that had received local gene delivery of mock or IL-22BP vector in the proximal colon. Protein lysates from the freshly isolated CECs were subjected to immunoblot with anti-phospho-STAT3 Abs (C). After stripping the Abs, the membrane was reprobed with anti-STAT3 Abs (C). The proximal colon of mock (left panel) or IL-22BP (right panel) gene-delivered mice was subjected to Alcian blue staining (D). Significant reduction of goblet cells (blue) is observed in the colon of IL-22BP-delivered mice as compared with that of mock-delivered mice. Ulceration is indicated by arrow. Original magnification, $\times 4$. Averages of goblet cells/field in the noninjected (distal colon) and injected (proximal colon) site are shown in E. Disease score in the proximal colon (injected site) is summarized in F. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

of DSS colitis, was observed in the control Ab-treated group but not the anti-IL-22 Ab-treated group (Figure 6D). In addition, intestinal injury as judged by disease scores was exacerbated in the anti-IL-22 Ab-treated group (Figure 6E). These findings suggest that IL-22 contributes to inflammation-associated goblet cell restitution.

Suppression of IL-22-mediated goblet cell restitution by IL-22-binding protein. IL-22-binding protein (IL-22BP) has been demonstrated to sufficiently neutralize IL-22 activity in vitro (38). Interestingly, expression of colonic IL-22BP was significantly downregulated during the recovery phase of DSS colitis (Figure 7A) at a time when IL-22 was upregulated (Figure 6A). Therefore, to further confirm an involvement of IL-22 in goblet cell restitution, local delivery of lipid complexes with secretion vector carrying a full-length mouse IL-22BP cDNA was performed in the proximal colon of WT mice. The mice were subsequently treated with DSS and then sacrificed 3 days after termination of the DSS treatment. Significantly, enhanced IL-22BP expression was observed in the injected, but not the noninjected, site of the colon (Figure 7B). IL-22BP gene delivery downregulated the activation of STAT3 in CECs within the injected, but not the noninjected, sites of the colon (Figure 7C). Of note, goblet cells were significantly reduced within the injected site of the IL-22BP-delivered group as compared with the mock-delivered group (Figure 7, D and E). In contrast, there was no significant difference in the goblet cell numbers within the noninjected sites among these groups (Figure 7E). Interestingly, ulcerations were recognized in the injected sites of all mice (6 of 6) that received IL-22BP gene delivery and 2 out of 6 mice that received mock delivery (Figure 7D). In addition, the inflammation in the proximal colon (injected site) was significantly exacerbated by IL-22BP gene

delivery (Figure 7F). These data suggest that IL-22BP suppresses inflammation-associated goblet cell restitution and recovery from acute intestinal injury by inhibiting IL-22 activity.

Discussion

IL-22 possesses the ability to induce both STAT3-mediated expression of regulatory molecules (7–10) and ERK-mediated expression of proinflammatory molecules (e.g., IL-8) (8, 11). The opposing effects of IL-22 have made it difficult to predict the role of IL-22 in IBD. We herein demonstrate that IL-22 contributes to rapid amelioration of local inflammation associated with a Th2-mediated colitis through activation of STAT3 in CECs. STAT3-mediated activation of acquired immune responses is well known as playing a pathogenic role in colitis by enhancing the survival of pathogenic T cells (37, 39). In contrast, STAT3-mediated activation of innate immune responses contributes to the suppression of colitis, as indicated by the spontaneous development of colitis in mice in which STAT3 is extinguished only in innate cells such as CECs or macrophages (40–42). Since IL-22 specifically targets innate immune pathways (7, 9), selective activation of STAT3 in CECs but not acquired immune cells by IL-22 may contribute to amelioration of chronic Th2-mediated colitis.

IL-22 has previously been demonstrated to stimulate keratinocytes and CEC lines to produce several regulatory molecules, such as IL-10, SOCS3, and antibacterial peptides (β -defensin 2, psoriasin, calgranulins A and B) (7–10). In addition, we herein demonstrate what we believe is a novel function of IL-22. IL-22 contributes to the improvement of colitis-associated mucus layer destruction associated with goblet cell depletion by enhancing the



production of membrane-bound mucins (Muc1, -3, -10, and -13). Membrane-bound mucins form a static external barrier at the epithelial surface and are stored in goblet cell vacuoles (13, 34, 43). Importantly, our studies suggest that the enhanced mucus barrier formation participates in the IL-22-mediated attenuation of Th2-mediated colitis. Indeed, recent studies have indicated that Muc2 and Muc3 contribute to the suppression of experimental colitis (33, 34). In addition, a more recent study clearly demonstrated a critical role of intestinal mucus layer for the suppression of colitis (44). UC, which is characterized by a thin mucin layer in association with goblet cell depletion, exhibits strong expression of Muc2 and Muc4 and low expression of Muc1 and Muc3 (13). Therefore, it is possible that insufficient production of IL-22 may facilitate goblet cell depletion and impair mucus layer formation in UC. Goblet cells specifically produce not only Muc but also other molecules involved in both regulation (e.g., trefoil factor) (45) and exacerbation (e.g., resistin-like β [RELM β]) (46) of colitis. Interestingly, IL-22 downregulated the expression of goblet cell-derived RELM β , a potential pathogenic goblet cell product (K. Sugimoto, unpublished observations).

Accumulating data in experimental IBD models have suggested the involvement of both common and distinct mechanisms of pathogenesis in UC versus CD. Experimental chronic UC-like disease is significantly contributed to by Th2 cytokines (14, 18). In contrast, IL-23/IL-17 pathways have recently been shown to play a pathogenic role in experimental CD-like diseases (15, 16, 47, 48). The relevance of the data from experimental colitis models to human CD is supported by recent studies showing a negative association of IL-23 receptor polymorphisms to the development of IBD (49). Interestingly, IL-22, which is preferentially expressed by Th17 cells (4, 5), is significantly increased in CD patients in comparison with UC patients (8, 11). Thus, Th17 cells may be responsible for both deleterious effects through IL-17 production and beneficial effects through IL-22 production through an increase of mucus production. Indeed, a recent study has demonstrated a contribution of the Th17 subset in the suppression of a Th2-mediated asthma model that is characterized by dysregulated mucus production in the trachea (50).

We developed a novel microinjection-based local gene-delivery system that allowed the targeting of inflamed mucosa and supplementation of local IL-22 expression restricted within the delivered site. Microbial-mediated *in vivo* gene delivery using adenovirus or *L. lactis* has been used to cause local overexpression of some molecules in the intestine (22, 23). However, it may be difficult to avoid certain risks in these approaches, e.g., unwanted immune stimulation by virus-like particles or expression of genes of interest throughout the entire intestine by enteric bacteria-mediated gene delivery. Alternatively, our microinjection-based gene-delivery approach has several advantages. These include the restricted expression of the gene of interest to the injection site, transient expression (less than 4 weeks), and utilization of potentially harmless carriers as compared with microorganisms. Therefore, it is likely that potential side effects are minimized. In humans, such microinjection-based local gene delivery may be performed via endoscopy.

In summary, we provide an unexpected insight into the role of the Th17 cytokine IL-22 in Th2-mediated chronic colitis of TCR α KO mice. IL-22 stimulates mucus production and goblet cell restitution under intestinal inflammatory conditions and also contributes to the rapid attenuation of this inflammation. In addition, we describe

a newly established local gene-delivery approach that is capable of targeting inflamed mucosa and may provide a means to developing a new therapeutic strategy for treating mucosal inflammation.

Methods

Reagents. Abs against phosphorylated (Thr202/Tyr204) and total ERK1/2 and phosphorylated STAT3 (Tyr705) (Cell Signaling); anti-STAT3, anti-MUC1, and anti-actin (C-II) Abs (Santa Cruz); anti-human β -actin Abs (Sigma-Aldrich); and HRP goat anti-rabbit and goat anti-mouse secondary Abs (Pierce) were used. Recombinant human IL-22 and recombinant mouse IL-22 were obtained from R&D Systems. Anti-IL-22 Abs that recognize a binding site within IL-22 to IL-10R2 (36) were generated by immunization of rabbits with keyhole limpet hemocyanin-conjugated peptides (VLLPQSDRFQPYMQE-c). The Abs were purified from the immune serum by affinity purification with the peptide used for immunization. The specificity of the Abs was confirmed by Western blot analysis using recombinant mouse IL-22 protein.

Mouse and human materials and disease evaluation. Mice were maintained under specific pathogen-free facilities at Massachusetts General Hospital. All mice used were of the C57BL/6 background. Viable and pure mouse CECs were isolated as crypt units by using an EDTA perfusion method, as previously described (37). Intestinal inflammation in mice was evaluated according to previously described criteria (28, 37). In chronic colitis, the disease score was estimated by a combination of gross score (0, normal appearance; 1, focal change; 2, mild change in entire colon; 3, severe change in entire colon) and histological scores (0–3, inflammatory cell infiltration; 0–3, epithelial cell elongation). In DSS-induced acute intestinal damage, ulceration score (0, no ulceration; 1, presence of erosion; 2, presence of focal ulceration; 3, presence of multiple ulcerations) was used instead of gross score. All experiments were approved by Subcommittee on Research Animal Care of Massachusetts General Hospital. Human colonic tissue samples were obtained from the tissue bank of the Center for the Study of Inflammatory Bowel Disease and Pathology at Massachusetts General Hospital. Caco2, SW480, and T84 cell lines were obtained from ATCC. T84 cells were grown on filter membranes until they formed a confluent polarized monolayer ($>1000 \Omega\text{-cm}^2$). Cells were then stimulated with several doses of cytokines from upper or lower compartments.

Gene expression analyses. RNA was extracted from the total mouse colonic LP after removing CECs by the EDTA perfusion method (30 mM), as previously described (21). 10 μg mRNA was subjected to DNA microarray analysis using PGA Mouse v1.0 probe set (19,549 oligos), as previously described (21). For the supplementation of genes that were not included in the gene chips used, large numbers of primer sets for the detection of 1,300 molecules were generated and used for real-time PCR analysis. Real-time PCR was carried out using the SYBR green system (Stratagene), as previously described (37).

Protein and mucus layer analyses. Western blot analysis was performed using the ECL detection system (Amersham), as previously described (37). Paraffin-embedded tissue sections were stained with H&E, Alcian blue, and PAS using standard techniques. For analysis of the mucus layer, Carnoy solution was used for the fixation of tissues to preserve the mucus layer (51). Immunohistochemical staining using frozen tissues was performed as previously described (21).

Gene silencing *in vitro*. To determine whether STAT3 is required for the IL-22-mediated enhancement of mucus-associated protein production, we initially optimized the conditions in order to sufficiently inhibit STAT3 expression in the T84 cell line by employing a Nucleofector approach to introduce combined STAT3-shRNA vectors. STAT3 knockdown was performed using STAT3 shRNA vectors (pKD-STAT3-v2 and -v3; Upstate). STAT1 knockdown was performed using STAT1 shRNA vectors (pKD-STAT1-v1 and -v2; Upstate). T84 cells were resuspended at 2×10^6 cells/ml



in Cell Line Kit T buffer (VCA-1002; Amaxa Biosystem) and subjected to nucleofection with 2 µg of shRNA or mock vector using Nucleofector (Amaxa) according to the manufacturer's instruction. One day after the transfection, cells were stimulated with 10 ng/ml of IL-22 for 2 days and immediately subjected to protein lysis and RNA extraction.

Local in vivo gene delivery. Full-length mouse IL-22 and IL-17A cDNAs from WT LP cells treated with DSS was amplified by RT-PCR (forward, 5'-CGATCTCTGATGGCTGTCT, reverse, 5'-ACGCAAGCATTCTC-CAGAGA for IL-22; forward, 5'-AACATGAGTCCAGGGAGAGC, reverse, 5'-CTGCCTGGCGGACAATCGAG for IL-17A) and cloned into pCR 2.1 TOPO vector (Invitrogen). After digestion with *Bam*HI and *Xho*I, cDNA was subcloned into the pIRES-hrGFP2 vector (Stratagene) or pSecTag2/Hygro vector (Invitrogen). For in vivo local gene delivery into the colon, DNA/lipid complex was made by the incubation of vectors with cationic lipid (DOTAP-cholesterol) with an enhancer 2 (in vivo MegaFectin reagent; Qbiogene) according to the manufacturer's instructions. After dilution of the lipid/DNA complex with 10 mM HEPES, the complex (20 µg DNA) was directly injected into the proximal colon of mice using a microinjection needle (Harvard Apparatus) through laparotomy. Selected TCRαKO mice that had developed severe colitis (the severity of colitis was evaluated by measuring the colonic diameter through the laparotomy procedure) were used as previously described (28). The mice receiving local gene delivery were sacrificed at the time points indicated in figure legends. For continuous mucolytic treatment, an ALZET osmotic pump (Alzet Co.) filled with *N*-acetyl-L-cystein was implanted within the dorsa of mice as previously described (52). *N*-acetyl-L-cystein (20 µg/h) was continuously delivered into the cecal lumen for 14 days through a catheter from the implanted osmotic pumps.

Inhibition of IL-22 activity in vivo. For DSS colitis, mice were treated with 3.5% DSS (MP Biomedicals) in drinking water for 5 days, and this treatment was terminated by changing DSS water to normal water. Repeated treatments with anti-IL-22 Abs or control IgG (1.5 mg/injection, 4 times)

at days 4, 5, 6, and 7 were performed in selected mice, which showed 3% to 5% of body weight loss at day 4 as compared with initial body weight. In some experiments, local gene delivery with pSecTag2/Hygro vector carrying full-length mouse IL-22BP cDNA was performed in the proximal part of the colon, and the mice were subsequently treated with 3% DSS on the third day after gene delivery. DSS treatment was terminated at day 5 after initiation of DSS treatment, and mice were sacrificed at day 8.

Statistics. Statistical analysis was performed using the Mann-Whitney U test. *P* values of less than 0.05 were considered statistically significant.

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